to study channels and transporters. We have combined the DIB with electrowetting on dielectric (EWOD) in order to move, join and separate the droplets under computer control, and thus be able to create complex and dynamic networks. The geometry and complexity of these droplet networks are dictated by the microfabricated network of electrodes used to move the droplets and to measure the current produced by ions flowing through the transporters inserted into the bilayer.
We built a prototypical chip where aluminium electrodes control droplet movement and gold electrodes, terminated by Ag/AgCl spots, apply a potential between the two droplets and measure the current passing through the membrane. We confirmed the feasibility of such studies by measuring current flowing through bacterial channels inserted into a DIB. We are now turning toward more complex networks and the study of eukaryotic proteins synthetized by cell-free techniques within the droplets. Preliminary results on this will be presented.
[1] Funakoshi K *et al.* (2006) *Anal Chem***78**: 8169-8174
[2] Bayley H *et al.* (2008) *Mol BioSyst***4**: 1191-1208

1337-Pos Board B247
Detection of Substrate-Dependent Conformational Changes in HP1 of the Glutamate Transporter GltPh
Derek J. Francis, David S. Cafiso, Joseph A. Mindell.
Glutamate acts as the primary excitatory neurotransmitter in the mammalian central nervous system. Clearance of this neurotransmitter from the synapse is accomplished by a family of glutamate transporters known as EAATs, which co-transport sodium and glutamate across the membrane. Recently, crystal structures of a related bacterial transporter, GltPh, have been solved. These structures have provided clues to the conformational changes associated with inhibitor binding as well as a large conformational change involved in switching the protein from an ''outward facing'' state to an ''inward facing'' state. However, crystallography has failed to provide data regarding the effects of sodium on the local structure of the substrate binding site. The details of how the substrate is released from the transporter are also absent. Finally, it remains undetermined which conformation(s) the protein adopts in physiological environments, and how the binding of sodium and substrate are coupled to conformational exchange. To probe these questions, we utilize the technique of site-directed spin labeling (SDSL) electron paramagnetic resonance (EPR) spectroscopy to explore the local structure and dynamics of residues within a helical hairpin (HP1) which has been suggested to serve as an intracellular gate. EPR spectra of labeled mutants describe a conformational change within HP1 upon addition of sodium and/or aspartate. The spectral changes are localized to the tip of the hairpin, suggesting a relatively small conformational change. In order to fully interpret these structural changes, we are currently measuring the distance between doubly-labeled residues to ascertain the global conformation (outward- vs. inward-facing) of the protein. Preliminary data suggest that in vesicles, GltPh is able to adopt a conformation which resembles the most recently solved crystal structure, which has been interpreted as an inward facing form of the transporter.

1338-Pos Board B248
Antibiotic Binding and Dynamics within the OmpF Channel Allow Transfer Across the Bacterial Outer Membrane
Brigitte Ziervogel, Balasundaresan Dhaksnamoorthy, Lydia Blachowicz, Benoit Roux.
Outer membrane protein F (OmpF) is a nonspecific, water-filled channel that allows the passage of hydrophilic antibiotics across the lipid barrier of Gram-negative bacteria. The diffusion limit for translocating molecules is provided by the constriction zone, which forms both a steric and an electrostatic hindrance due to a unique arrangement of charged residues that reach into the pore. Antibiotic-resistant bacterial isolates have been found that show either reduced expression of OmpF protein or mutation of key constriction zone residues. Therefore, it is necessary to identify important drug-protein molecular interactions that facilitate efficient transfer across the OmpF channel. We have co-crystallized E. coli OmpF with zwitterionic ampicillin, anionic carbenicillin, and bulky enrofloxacin, and observe the density corresponding to the antibiotic molecules inside the channel. Comparison of the experimental x-ray density with molecular dynamics simulations allows multiple conformations of the diffusing molecule to be resolved. Results of this work give insights into how the charge distribution and size of the translocating molecule affect binding interactions and dynamics within the OmpF constriction zone. We are also using novel computational methods to model transition pathways for various molecules across the entire length of the porin for both WT and mutant OmpF proteins. Together, this work will assist in the design and screening of new antibiotics with improved diffusional characteristics. [Supported by NIH grant GM062342].

1339-Pos Board B249
pH-Sensitive Monomer-Dimer Equilibrium and Self-Association of the Cytoplasmic, N-Terminal Domain of the NBCe1-A Cotransporter
Harindarpal Gill.
NBCe1-A is an integral membrane protein that cotransports Na+ and HCO3- ions across the basolateral membrane of the proximal tubule. It is essential for maintaining a homeostatic balance of cell and blood pH. In X-ray diffraction studies, we reported that the cytoplasmic, N-terminal domain of NBCe1-A (NtNBCe1-A) is a dimer. Here, MALS-SEC and DLS measurements show that NtNBCe1-A has three additional states in solution that are characterized by its molecular masses, stability, and hydrodynamic properties as a function of pH. Firstly, NtNBCe1-A molecules in solution are dimers in equilibrium with monomers. Monomer formation increases as pH gradually rises to pH 11.5. This dimer disassociation is supported further by intrinsic tryptophan-fluorescence and circular-dichroism spectroscopy studies that demonstrate a gradual Stoke's shift of 14 nm from pH 6.5 to 11.5 without significant alteration to the secondary structure. Secondly, NtNBCe1-A molecules also form prominent clusters of molecular masses 3-5 times that of a dimer. This clustering is reversible and is further supported by surface plasmon resonance (SPR-Biacore) techniques that illustrate NtNBCe1-A molecules transiently self-associate most prominently in the range of pH 7 to 7.4. Association and dissociation rates will be reported. Thirdly, at high pH and protein concentration, NtNBCe1-A molecules slowly and randomly self-associate into unstable, high-order aggregates. Taken together, a hypothetical model is presented where the Nt acts as a pH sensor inside the cell and plays a role in self-association of NBCe1-A molecules in the membrane.

1340-Pos Board B250
The Cytoplasmic Ion Permeation Pathway of the Cardiac Na^+-Ca^{2+} Exchanger
Michela Ottolia, Debora A. Nicoll, Kenneth D. Philipson.
The Na^+-Ca^{2+} Exchanger (NCX) is a plasma membrane protein that exchanges three Na^+ for one Ca^{2+}. Transport function has been associated with two regions of NCX that have similar sequence: the α-repeats. Mutations here alter the transport properties of NCX. These two regions, which consist of residues spanning TMSs 2 and 3 (α-1 repeat) and a portion of TMS 7 with the following intracellular loop (α-2 repeat), are modeled to be in close proximity and may form an ion conduction pathway. The goal of this work was to understand transport function and define the pathway through which Na^+ and Ca^{2+} diffuse from the cytoplasm to their intracellular binding sites. We mutated TMS 2 residues to cysteine and determined the affinities of these mutants for Na^+ and Ca^{2+}. Also, the reactivity of cysteines to MTSET was investigated to demonstrate intracellular accessibility. Of mutations at 10 positions, 3 behaved like the wild-type exchanger (A106C, P112A, S117C). Mutant M105C had altered affinity for Na^+ but not Ca^{2+} and, like A106C, was blocked by cytoplasmic MTSET in the presence or absence of Na^+. In contrast, Na^+ blocked the sensitivity of L107C, S109C and S110C to MTSET. This suggests that Na^+ binding prevents the cytoplasmic accessibility of these residues. Additionally, mutations at L107, S109 and S110 had altered affinities for intracellular Na^+ and Ca^{2+}. For S110C, the selectivity properties of NCX were also altered allowing Li^+/Ca^{2+} exchange. We conclude that TMS 2 is involved in ion binding and transport and is accessible to cytoplasmic sulfhydryl agents in a substrate-dependent manner. The data also suggest the presence of an aqueous cavity used by Na^+ and Ca^{2+} to reach their binding sites involving TMS 2.

1341-Pos Board B251
Insights into the Mechanism of Release of Substrate in the Inward Facing Glutamate Transporter from Molecular Dynamics Simulations
Indira Shrivastava, Jason DeChancie, Ivet Bahar.
Glutamate transporters regulate neurotransmission across glial and neuronal cell membranes, by actively coupling to the energetically favorable transport of cations including sodium or potassium and/or protons. Previous simulations of the outward facing glutamate transporter GltpH from Pyrococuss Horikoshii (PDB ID 1xfh) (Shrivastava et al, J. Biol. Chem. ,2008), revealed the mechanism of substrate diffusion and binding at the binding site. The binding was facilitated by the intrinsic dynamics of the extracellular gate, comprised of a hairpin loop, HP2. In the inward facing structure of the same protein, (PDB ID 3KBC) the transport machinery comprising of TM7, HP1,HP2 and TM8 is translated by almost 15 Å[[Unsupported Character -]]downwards towards the intracellular side, with respect to the outward facing structure. Fifty nanosecond simulations each, of the Apo_MD (no substrate or sodium ions in the core), Na_MD (only sodium ions in the core) and the Asp_Na_MD

(substrate and sodium ions in the core) were performed, in a fully atomistic, solvated bilayer environment. In the Apo_MD and the Na_MD simulations, the HP1 loop has a much higher mobility than the HP2 loop. However, the release of the substrate into the intracellular solvent in Asp_Na_MD, required the motions of both the HP1 and the HP2 loops. The opening up of HP2 loop facilitates solvation of the binding site resulting in the substrate being dislodged from its position. Prior to substrate release, the HP1 loop moves further down into the solvent, exposing the HP1-tip and the substrate to the solvent, followed by its subsequent release into the intracellular solvent. These results suggest that the intracellular gating involves sequential opening of both HP1 and HP2 loops.

1342-Pos Board B252

Ligand Exit and Entry Pathways for Monoamine Transporters

Bonnie A. Merchant, Jeffry D. Madura.

The monoamine transporters are targets for various medicinal and illegal drugs that affect mood and behavior. Of particular interest are the dopamine (DAT) and serotonin (SERT) transporters of which the three-dimensional structures are unknown. A three-dimensional structure homologous to DAT and SERT, both in sequence and in function, is the leucine transporter ($LeuT_{Aa}$). While there is significant binding and uptake data, some structural information and homology models, there is no clear understanding of the transport pathways for ligands of $LeuT_{Aa}$, DAT or SERT. The Random Acceleration Molecular Dynamics (RAMD) method as implemented in NAMD, was used to study the entry and exit pathways of various chemically relevant substrates in $LeuT_{Aa}$ and a homology model of DAT. Example pathways as illustrated in Figure 1. Free energy scores of the pathways have been characterized via the Multi-Configuration Thermodynamic Integration method. Several sites of low free energy score have been indentified, which correspond to primary and secondary substrate pockets of the transporters. Detailed free energy and structural results of the transport pathways will be presented.

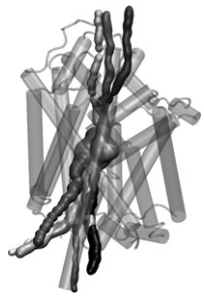

Figure 1. Representative transport pathways of leucine through LeuTAa using RAMD.

1343-Pos Board B253

Proton Transport and Conformational Changes in H+/CL- Exchangers

Mattia Malvezzi, Alessandra Picollo, Alessio Accardi.

CLC transporters localize to the membranes of intracellular compartments, such as lysosomes and endosomes, where they mediate a variety of physiological roles by exchanging 2 Cl^- ions for 1 H^+. Very little is known about the mechanisms underlying the transport process and many questions are still unanswered, in particular the H^+ pathway has not been identified and which conformational changes the transporters undergo is still not clear. To elucidate these important aspects we decided to investigate how protons translocate through the protein by studying the deuterium kinetic isotope effect and to probe the conformational changes by measuring the temperature dependence of the transport rate of the human transporter CLC-5 and the bacterial homologue CLC-ec1. We found that both CLC-5 and CLC-ec1 have similar thermodynamic profile. The transport rate in deuterium is decreased by ~20-40%, suggesting a H^+ movement through a hydrogen-bonded pathway, possibly formed by water. Both transporters also have a similar and modest temperature dependence, suggesting that the proteins undergo limited conformational changes. Interestingly, we observed unaltered apparent activation enthalpy of transport when Cl^- or H^+ binding, coupling or transporter gating are impaired, while the transport rates are affected. We hypothesize that H^+ movement through a CLC transporter takes place along a series of hydrogen bond formed by water molecules and that only limited conformational changes occur during the transport cycle. Finally, we propose a transport mechanism where several rate-limiting steps with similar apparent activation enthalpies are involved, instead of a single rate-limiting step mechanism.

1344-Pos Board B254

Analysis of the Oligomeric State of Surface-Localized Proton-Coupled Folate Transporter by Blue Native Polyacrylamide Gel Electrophoresis

Prachi Nakashe, Michaela Jansen.

Folate vitamins are essential for DNA replication and cellular proliferation. However, mammalian organisms are devoid of de novo folate biosynthesis and thus rely on dietary sources to meet their metabolic needs. The proton coupled folate transporter (PCFT/SLC46A1) has been recently identified as the molecular entity of the carrier mediated intestinal folate uptake pathway for folic acids from food sources. PCFT is also involved in the absorption of chemotherapeutically used antifolates. Currently, there is limited information about the structure and function of PCFT. Hydropathy analysis suggests that there are 10-12 transmembrane segments. Further, using the Substituted Cysteine Accessibility Method (SCAM) evidence was provided for a 12 transmembrane segment topology. Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) is a technique for separation of protein complexes in a native state with high resolution. We expressed PCFT in Xenopus laevis oocytes. Oocyte plasma membranes were polymerized to the vitelline membrane using ludox colloidal silica solution and polyacrylic acid, isolated by centrifugation, and plasma membrane proteins subsequently solubilized with digitonin and separated by BN-PAGE. The separation characteristics of native PCFT were compared to a molecular ruler produced by partial dissociation of homopentameric 5-hydroxytryptamine type 3A (5HT3A) receptors. Under native conditions, 5HT3A subunits largely migrated as a pentamer and PCFT only as a monomer. Treatment with denaturing agents generated a ladder of five bands for 5HT3A subunits, which consisted of monomer, dimer, trimer, tetramer and pentamer. Addition of crosslinking agents resulted in migration of 5HT3A subunits as a pentamer, even in the presence of denaturing agents. In contrast, crosslinking agents did not induce oligomeric assemblies of PCFT. These results indicate that functional plasma-membrane bound PCFT is a monomeric protein.

1345-Pos Board B255

Sets of Local Entropy-Enthalpy Change Leads to Global Entropy-Enthalpy Change in SERCA

Anu Nagarajan, Thomas B. Woolf.

Major conformational changes are involved in the multi-step catalytic cycle of the sarcoplasmic endoplasmic reticulum calcium ATPase (SERCA) pump. The movement and rotation of the Actuator (A) domain is crucial for ion translocation. The A-domain is connected to the trans-membrane helices through linker regions. Mutational studies on the A-M3 linker region show that varying the length of this region causes significant changes in the rate of the conformational transitions (JBC (2009),284,12258-12265). The focus of this research is to study the impact of these mutations on the structural changes during the transition from E1 to E2, the behavior of the A-M3 linker region, and the overall rate of the conformational transitions. In order to achieve faster computation of transitions, we implemented the MARTINI coarse-grained protein and lipid model in CHARMM. We used Dynamic Importance Sampling (DIMS) to compute transitions from the E1 to the E2 state for both directions of each mutant in both coarse-grained and all atom models. Analysis of the transitions across mutants shows that the angle formed by A-P-N domains changes by up to 20 degrees with an increasing number of inserts into the A-M3 linker region. Estimates of barrier crossing time from the simulation and experimental values are highly correlated (R^2=0.934). Quasiharmonic analysis on the domains, linkers and transmembrane helices show entropic changes between the mutants and compensation effects. Interaction energies of the same regions indicate entropic-enthalpic compensation. Further investigation of the end state simulations shows changes in the number of high density water sites around the A-M3 linker region across the mutants. The varying degree of the change in volume due to water sites across all the mutants indicates a ripple effect where the local entropy-enthaply changes translate to global entropy-enthalpy changes.

1346-Pos Board B256

Functional Analysis of Transmembrane Domain 3 in NKCC1

Suma Somasekharan, Biff Forbush.

We sought to determine the functional roles of residues in transmembrane domain (TM) 3 of human NKCC1 using tryptophan and cysteine scanning mutagenesis. We generated a structural alignment of the transmembrane domains of NKCC1 to the related APC transporters ApcT and AdiC, and obtained 3-D alignments using Modeller. Based on these alignments, residues 368 to 380 were predicted to form part of the inner 2/3 of the translocation pore. We substituted these residues with tryptophan and cysteine and determined the impact of the changes on protein synthesis and cell surface delivery by Western blotting and immunofluorescence microscopy. Most of our mutants expressed and localized similar to wild type NKCC1 and these were analyzed in depth for transporter function by means of Rb^{86} influx assays. A working hypothesis is that tryptophan mutants that are much reduced in function are too hydrophobic for the solvent interface and those residues that retain function are either protein interior or lipid facing. The pattern of the tryptophan scan followed an alpha helical periodicity. Based on the tryptophan scan we deduced that the non-functional mutants I368W, G369W, F372W, A375W, N376W, A379W are pore lining residues. Cysteine scanning complemented the results of the tryptophan scan. Since cysteine is a mild mutation, most mutants were functional. However I368C, G369C, A379C showed dramatic reduction or loss of function